

Heterogeneous nuclear ribonucleoprotein A3 binds single-stranded telomeric DNA and inhibits telomerase extension *in vitro*

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Abstract

Telomeres are dynamic DNA–protein complexes at the end of linear chromosomes. Maintenance of functional telomeres is required for chromosome stability, and to avoid the activation of DNA damage response pathway and cell cycle arrest. Telomere-binding proteins play crucial roles in the maintenance of functional telomeres. In this study, we employed affinity pull-down and proteomic approach to search for novel proteins that interact with the single-stranded telomeric DNA. The proteins identified by two-dimensional gel electrophoresis were further characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF-TOF tandem MS. Among the five identified proteins, we report here the biochemical properties of a novel protein, hnRNP A3. The purified hnRNP A3 bound specifically to G-rich strand, but not to C-rich strand or double-stranded telomeric DNA. The RRM1 (RNA recognition motif 1) domain, but not RRM2, of hnRNP A3 is sufficient to confer specific binding to the telomeric sequence. In addition, we present evidence that hnRNP A3 can inhibit telomerase extension *in vitro*. These biochemical properties of hnRNP A3 suggest that hnRNP A3 can participate in telomere regulation *in vivo*. © 2007 Elsevier B.V. All rights reserved.

Keywords: Telomere; Telomerase; hnRNP A3; Telomere-binding protein; RNA recognition motif

1. Introduction

Telomeres are specialized structures at the ends of chromosomes. Human telomeric DNA consists of several thousands of tandem-repeated sequences with a short single-stranded overhang on the 3' G-rich strand. A complex formed with six telomere-specific proteins, which include POT1, TRF1, TRF2, Rap1, TIN2 and TPP1 [1,2], associates with the terminal region to provide a protective cap and allows cells to distinguish telomeres from DNA breaks [3–5]. Although the precise structure of telomeres *in vivo* is not known, it has been suggested that telomeres may exist as dynamic structures between capped or uncapped states [6,7]. An uncapped telomere may contain a 3' overhang of G-rich strand. On the other hand, a t-looped structure of telomere has been observed *in vitro* and from chro-

matin isolated from human and mouse cells [8], and is postulated to serve as the capped structure that protects telomere from DNA degradation and unwanted recombination.

Functional telomeres play an important role in cellular senescence [4,7,9]. Dysfunctional telomeres are known to trigger growth arrest, senescence, apoptosis, and an increase of end-to-end fusions and anaphase bridges [4,7]. Dysfunctional telomeres may be produced via various mechanisms. Telomere attrition represents a normal mechanism that limits the replicative potential of human somatic cells and can serve as a tumor suppressor pathway for potential cancer cells. The gradual loss of telomeric DNA with each round of DNA replication depletes the telomere reserve and leads to a growth arrest that is accompanied by senescence or apoptosis, a process known as replicative senescence. Telomere dysfunction can also occur due to loss or mutation of telomere-binding proteins. Loss of TRF1 or TRF2 function had been shown to lead to chromosomal abnormalities, cell cycle arrest, and the activation of DNA damage response pathway [7,10].

The most versatile and widely used method of telomere length maintenance is based on telomerase. Human telomerase is

Abbreviations: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; hnRNP, heterogeneous nuclear ribonucleoprotein; RRM, RNA recognition motif

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a specialized ribonucleoprotein polymerase that directs the synthesis of telomeric repeats (TTAGGG) at 3' ends of the G-rich strand [11]. Normal human somatic cells undergo telomere attrition because of inadequate level of telomerase. In contrast, a majority of immortal and cancer cells have an indefinite proliferative capacity and maintain their telomere length by upregulating telomerase [12,13]. While the regulated telomerase expression may serve as the primary control of telomere length maintenance, the synthesis of telomeric DNA by telomerase is anticipated to be affected by many factors such as the recruitment of telomerase to the telomere terminus, the initiation of elongation, and/or the processivity of the elongation cycles. Therefore, telomere length is influenced by the level of telomerase expression but also depends on control pathways that act on recruitment of telomerase and/or in *cis* at each individual telomere. Indeed, telomere-binding proteins and telomerase-interacting proteins have been demonstrated to participate in telomere length homeostasis [10,14,15].

In view of the important role of telomere-binding proteins in the telomere functions, we have employed affinity purification protocol and proteomic approach to search for novel proteins that bind to G-rich strand of telomeric DNA. Here, we report our biochemical studies of a newly identified telomere-binding protein, hnRNP A3. Based on the biochemical properties of

purified hnRNP A3 *in vitro*, we suggest that this protein can participate in telomere length maintenance *in vivo*.

2. Materials and methods

2.1. Chemicals, enzymes, oligonucleotides, and antibodies

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, Superscript III reverse transcriptase, TRIzol reagent, and antibiotics were from Gibco-BRL. RNase inhibitor was purchased from CalBiochem Co. Taq DNA polymerase was from Abgene, Epsom, UK. Polyclonal antibody against hnRNP A3 was prepared by Genesis Biotech. Inc. using oligopeptide VKPPPGRPQPD-SFRC. Gel electrophoresis reagents were from Bio-Rad. All other chemicals were from Sigma Chemical Co. The biotinylated and non-biotinylated oligonucleotides G-1 (GCCAAGGAGCCATTAGGG), G-2 (GCCAAGTTAGGGTTAGGG), G-3 (TTAGGGTTAGGGTTAGGG), G-4 (TTAGGGTTAGGGTTAGGGTTAGGG), C-3 (CCCTAACCCCTAACCCCTAA), G-3S (TAGTGGAGTTGGTGTGAG), and NS (GCCAAGGAGCCATGAGTG) were purchased from Bio Basic Inc. (Ontario, Canada). Double-strand telomeric DNA (dsG/C) was constructed by annealing G-3 and C-3. Other oligonucleotides were obtained from Bio Basic Inc.

2.2. Cell culture

Nasopharyngeal carcinoma-derived cell line NPC-TW02 (formally named NPC-076 in reference [16]) was routinely cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 0.25 µg/ml amphotericin B, and used as a convenient source

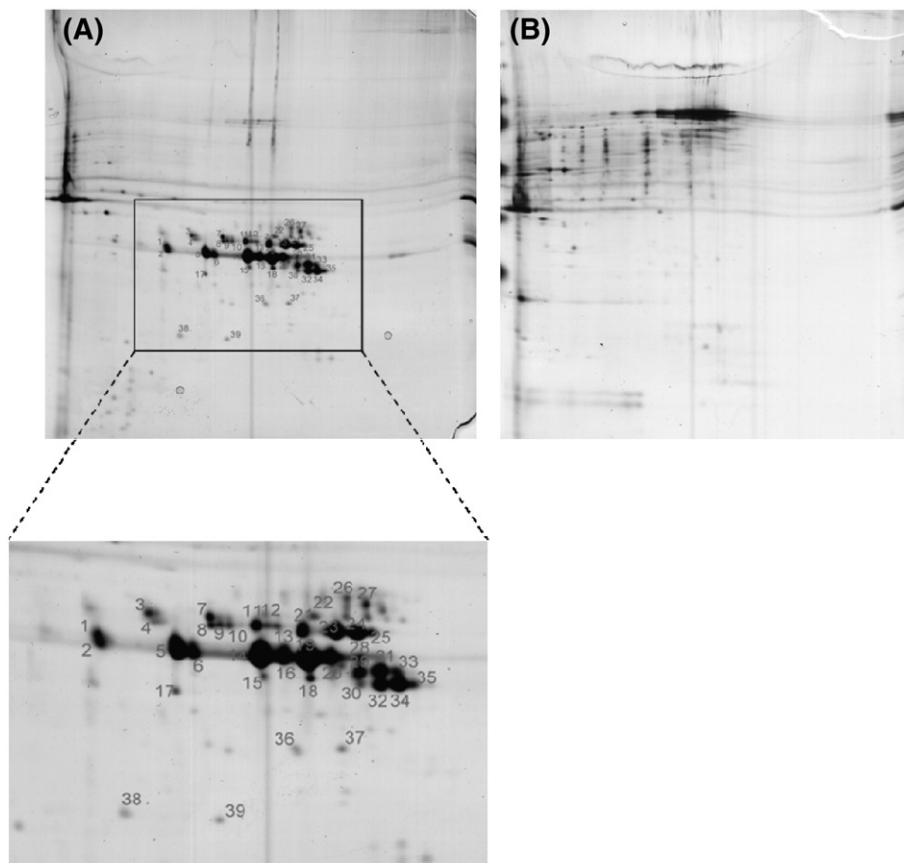


Fig. 1. Two-dimensional gel electrophoresis of proteins that bind to single-stranded telomeric DNA. Nuclear proteins were mixed with biotinylated G-3 (panel A) or biotinylated G-3S (panel B). Proteins that bound to the oligonucleotides were first electro-focused in a pH 6–11 gradient, and then separated in the second dimension by electrophoresis in a 12% SDS-PAGE. The proteins were identified by silver staining. The numbered protein spots in panel A were putative telomere-binding proteins and were further analyzed by MALDI-TOF MS.

for preparing nuclear extracts. Cells were grown at 37 °C in a humidified incubator containing 5% CO₂.

2.3. Nuclear extracts

Cells were suspended in NEBA buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), and NP-40 was added to a final concentration of 0.6%. After vortexing for 10 min, the disrupted cells were centrifuged at 14,000 rpm for 1 min. The pellets were resuspended in 50 µl of AFB A 300 (20 mM HEPES, pH 7.9, 300 mM KCl, 1 mM EDTA, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF) and vortexed for 15 min at 4 °C. After centrifugation for 5 min at 14,000 rpm, the supernatants were collected and used as nuclear extracts. The protein concentrations were determined using Coomassie protein assay reagent (Bio-Rad).

2.4. Affinity purification of telomere-binding proteins

The general protocol for affinity purification followed that described by Schnapp et al. [17]. In brief, nuclear extracts (5 mg/ml) were mixed with biotinylated G-3 or G-3S in AFB A300 buffer containing 0.5% Triton X-100 and 50 µg/ml yeast tRNA. After incubating at 30 °C for 5 min, samples were mixed with pre-blocked NeutrAvidin beads (UltraLink Immobilized NeutrAvidin Plus,

PIERCE). After washing three times with AFB A300 containing 0.5% Triton X-100 and two times with AFB A300, samples were suspended in 100 µl of 2D lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris base) to dissolve the proteins bound to the beads.

2.5. Separation of proteins by two-dimensional electrophoresis

The proteins bound to the affinity oligonucleotides were placed in a pH 6–11 immobilized non-linear gradient (IPG) strip (Amersham Biosciences). The proteins in the IPG strip were isoelectrically focused as follows: 30 V, 12 h; 50 V, 0.5 h; 100 V, 0.5 h; 250 V, 0.5 h; 500 V, 0.5 h; 1000 V, 0.5 h; 4000 V, 0.5 h; and 8000 V, 48 h. After focusing, the IPG strip was immersed in a TG buffer [50 mM Tris–Cl, pH 8.8, 6 M urea, 30% glycerol (v/v), 2% SDS (w/v), 0.002% bromophenol blue] containing 2% DTT for 15 min, and then in TG buffer containing 2.5% iodoacetamide for 15 min. The buffer-balanced IPG strip was then placed onto a 10% SDS-polyacrylamide gel and electrophoresed with a constant 30 mA/gel for 3.5 h. The proteins on the gel were identified by silver staining.

2.6. Analysis of proteins by MALDI-TOF MS

The sliced gels containing the desired protein were washed with water and 50 mM NH₄HCO₃/acetonitrile (1:1), destained with a solution containing 6 mM

Table 1
Identification of proteins that bind to telomeric DNA

Spot	Identify of protein	Mass	PI	Coverage (%)	Accession no.	Score
1	Unidentified					
2	hnRNP A2/B1	36041/37464	8.67/8.97		AAB60650/B34504	95/92
3	hnRNP A3	39779	9.1	23	Q6URK	57
4	hnRNP A2/B1	36041/37464	8.67/8.97	43/41	AAB60650/B34504	95/62
5	hnRNP A2/B1	36041/37464	8.67/8.97	54/52	AAB60650/B34504	182/175
6	hnRNP A2/B1	36041/37464	8.67/8.97	49/48	AAB60650/B34504	137/33
7	SYNCRIP protein	46410	8.94	23	AAH40844	48
8	Unidentified					
9	Unidentified					
10	Carcinoma-associated Ag	35564	7.42	25	B48149	40
11	hnRNP A3	39779	9.1	34	Q6URK	79
12	hnRNP A3	39779	9.1	26	Q6URK	61
13	hnRNP A3	39779	9.1	29	Q6URK	52
14	hnRNP A2/B1	36041/37464	8.67/8.97	62/60	AAB60650/B34504	213/208
15	hnRNP A2/B1	36041/37464	8.67/8.97	48/47	AAB60650/B34504	100/96
16	hnRNP A2/B1	36041/37464	8.67/8.97	51/50	AAB60650/B34504	228/233
17	hnRNP A2/B1	36041/37464	8.67/8.97	43/42	AAB60650/B34504	83/80
18	hnRNP A2/B1	36041/37464	8.67/8.97	50/49	AAB60650/B34504	86/83
19	hnRNP A2/B1	36041/37464	8.67/8.97	41/49	AAB60650/B34504	242/235
20	hnRNP A2/B1	36041/37464	8.67/8.97	70/67	AAB60650/B34504	219/208
21	hnRNP A2/B1	36041/37464	8.67/8.97	41/49	AAB60650/B34504	140/154
22	hnRNP A2/B1	36041/37464	8.67/8.97	48/46	AAB60650/B34504	96/93
23	hnRNP A2/B1	36041/37464	8.67/8.97	64/63	AAB60650/B34504	247/240
24	hnRNP A2/B1	36041/37464	8.67/8.97	71/71	AAB60650/B34504	248/254
25	hnRNP A2/B1	36041/37464	8.67/8.97	53/54	AAB60650/B34504	161/169
26	hnRNP A1	38805	9.26	33	ROA1_HUMAN	52
27	hnRNP A1	38805	9.26	60	ROA1_HUMAN	202
28	hnRNP A1	38805	9.26	34	ROA1_HUMAN	60
29	hnRNP A1	34245	9.27	61	S02061	158
30	hnRNP A1	34245	9.27	54	S02061	115
31	hnRNP A1	34245	9.27	43	S02061	66
32	hnRNP A1	34245	9.27	37	S02061	54
33	hnRNP A1	34245	9.27	61	S02061	142
34	hnRNP A1	34245	9.27	61	S02061	163
35	hnRNP A1	34245	9.27	54	S02061	79
36	hnRNP A2/B1	36041/37464	8.67/8.97	46/48	AAB60650/B34504	95/102
37	hnRNP A2/B1	36041/37464	8.67/8.97	47/45	AAB60650/B34504	102/99
38	hnRNP A2/B1	36041/37464	8.67/8.97	35/37	AAB60650/B34504	66/72
39	hnRNP A2/B1	36041/37464	8.67/8.97	48/50	AAB60650/B34504	99/105

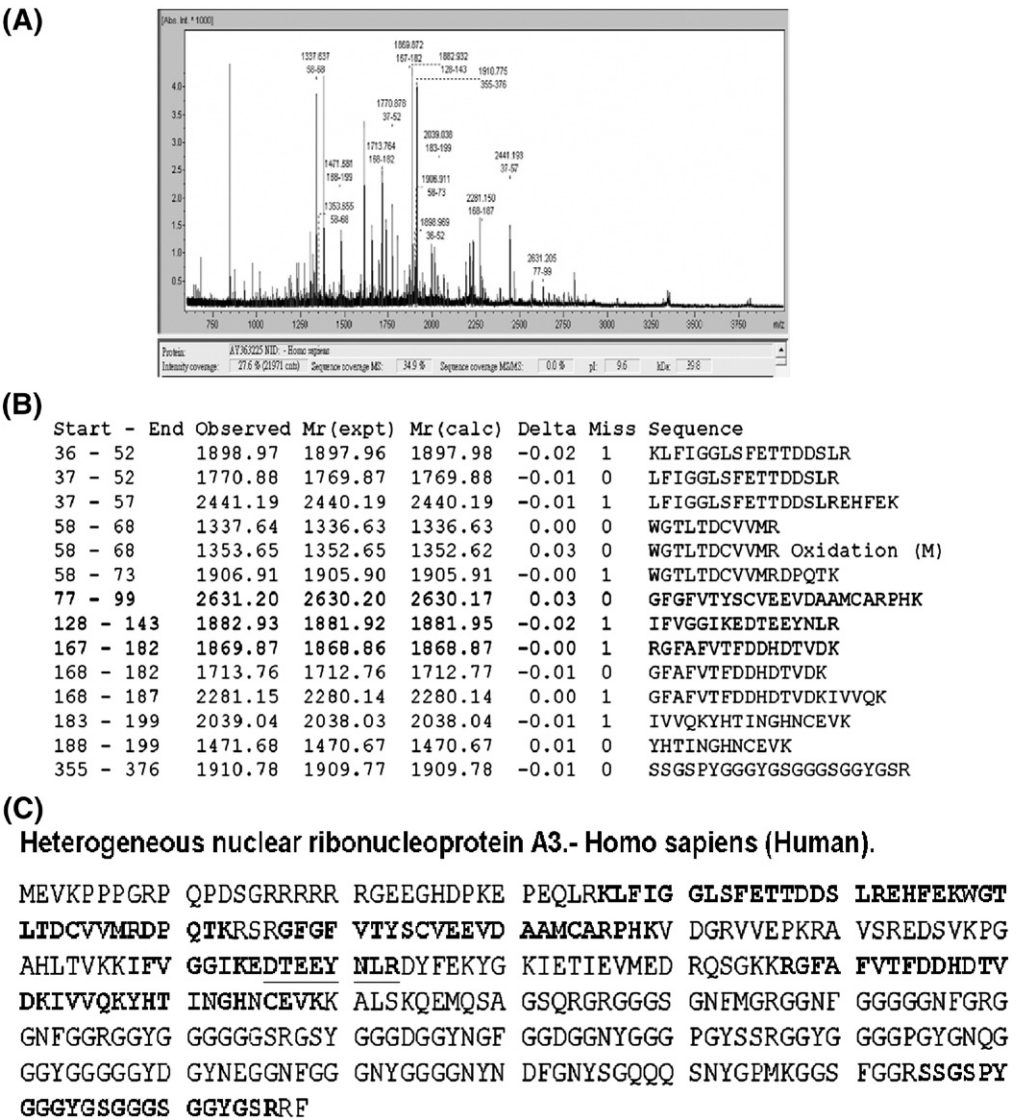


Fig. 2. Analysis of Spot 11 protein by MALDI-TOF spectrometry. The protein of Spot 11 in Fig. 1 was in-gel digested, and analyzed by MALDI-TOF spectrometry. The spectrum of peptides with different *m/z* is shown in panel A. The sequence of peptides (panel B) was deduced by searching MSDB database. The sequences that match human hnRNP A3 are shown as bold characters in panel C. The peptide with *m/z* 1882.93 was further analyzed by TOF–TOF and the deduced peptide is shown as underlined in panel C.

potassium ferricyanide and 1 mM sodium thiosulphate, and then immersed in 25 mM NH₄HCO₃. After becoming transparent, the gels were treated with acetonitrile before digesting with trypsin (10 µg/ml) at 37 °C for 12 h. The digested peptides in acetonitrile containing 0.5% trifluoroacetic acid were spotted on the sample tray, dried, and mixed with 0.2 µl matrix α-cyano-4-hydroxy cinnamic acid. The samples were analyzed on Ultraflex MALDI-TOF/TOF MS (Bruker Daltonics, Germany). The software Biotool was used to analyze the spectra obtained.

2.7. DNA cloning

Total mRNAs were extracted by TRIzol and were reverse transcribed (RT) by reverse transcriptase to generate cDNAs. For the cloning of hnRNP A3, the primers 5'-AACCATATGGAGGTAAACCCGCCG-3' and 5'-CTGGGATTC-TTAGAACCTTCTGCTACCATATCCAC-3' were used to amplify the cDNA by polymerase chain reaction (PCR). The conditions for RT-PCR were similar to those described earlier [16]. The DNA products of RT-PCR were purified and

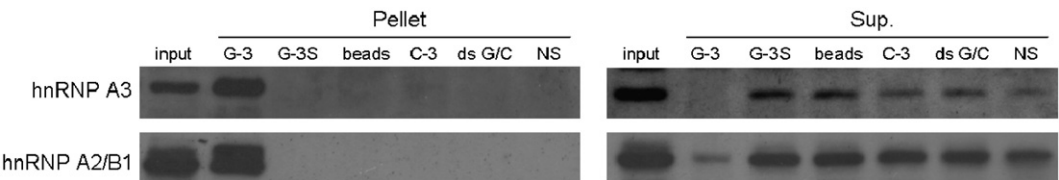


Fig. 3. Western blot analysis of the pull-down proteins. Nuclear proteins were mixed with biotinylated G-3, G-3S, C-3, NS, ds G/C or beads alone, and the presence of hnRNP A3 and hnRNP A2/B1 in the supernatant (Sup) or pellet (Pellet) was analyzed by Western blot.

cloned into vector pGEM-T (pGEM-T TA cloning kits, Promega) by following the manufacturer's suggestions. After transforming into *Escherichia coli* DH5 α cells, recombinant clones (pGEM-T/hnRNP A3) were obtained, and confirmed by restriction enzyme analysis and DNA sequencing. Subcloning of hnRNP A3 onto bacterial expression vector pET-15b (Novagen) was achieved by ligating the *NdeI/BamHI* digested DNA fragments from pGEM-T/hnRNP A3 to the *NdeI/BamHI* digested pET-15b DNA, and transforming into *E. coli* DH5 α cells. Cloning of the RRM1 (RNA recognition motif 1), RRM2 or RRM(1+2) domains of hnRNP A3 (see Fig. 4) was done by first cloning the desired DNA fragments into the pGEM-T vector and then subcloned onto the pET-15b as described above. The sets of primers used for the PCR amplification of the desired DNA fragments using pGEM-T/hnRNP A3 DNA as the template were M1-F (5'-AACCATATGGAGGTAACCGCCGCCGC-3') and M1-R (5'-CTGGG-ATCCTTAACCAGGCTTTACAGAATC-3') for RRM1; M2-F (5'-ACGCA-TATGGCCCATCTAACAGTGA-3') and M2-R (5'-CTGGGATCC-TTAACGACCTCTCTGTGATCC-3') for RRM2; and M1-F and M2-R for RRM(1+2).

2.8. Expression and purification of hnRNP A3 and its derivatives

The recombinant plasmids were transformed into *E. coli* strain BL21(DE3) pLysS. Following the induction with IPTG, the His-tagged proteins were purified through nickel column as described [18]. Western blot analysis was performed as previously described [16].

2.9. DNA-binding and telomerase assays

The DNA-binding activity of the purified proteins was assayed by both EMSA (electrophoresis mobility shift assay) and affinity pull-down methods. In the EMSA, biotinylated oligonucleotides were reacted with the purified proteins at 24 °C for 30 min in a buffer containing 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl₂, 50 ng/ μ l poly(dI:dC), and 0.05% NP-40. The reaction mixtures were electrophoresed in a native polyacrylamide gel, and then transferred to immobilon-NY+membrane (Millipore). Detection of biotinylated DNA in the membrane was done with LightShift Chemiluminescent EMSA kit (Pierce Co.) by following the manufacturer's suggestions. In the affinity pull-down assay, recombinant protein (5 μ g) was reacted with 0.1 nmol biotinylated DNA at 30 °C for 5 min in AFB A300 containing 0.5% Triton X-100 and 10 μ g/ml yeast tRNA. The proteins bound to the affinity DNA were pulled down by NeutrAvidin beads as described above in the section for Affinity purification of telomere-binding proteins. Telomerase was assayed by telomeric repeat amplification protocol (TRAP) as described previously [19].

3. Results

3.1. Identification of proteins that bind to single-strand G-rich telomeric DNA

The proteins that bound specifically to telomeric DNA were isolated by affinity purification using biotinylated G-3 as the affinity probe. In our initial trials, the isolated proteins were separated by 2D gel electrophoresis using a pH 3–10 IPG strip in the first dimension and 8% SDS-polyacrylamide gel in the second dimension. We observed that there were many poorly separated telomere-binding proteins with an expected pI around pH 8–9 and Mr around 35–45 kDa (data not shown). To improve the separation of these proteins, we have modified our 2D protocol using a pH 6–11 IPG strip in the first dimension and a 12% SDS-polyacrylamide gel in the second dimension. Representative results were shown in Fig. 1. The protein spots that were detected only from the biotinylated G-3 were marked. After in-gel digestion with trypsin, the peptides from these protein spots were analyzed by MALDI-TOF MS. The

putative identities of these proteins are summarized in Table 1. Out of the total 39 protein spots analyzed, no identity can be assigned to 3 spots (#1, 8, 9). The proteins in Spots 2, 4–6, 14–25, 36–39 were assigned as hnRNP A2/B1; the proteins in Spots 26–35 were assigned as hnRNP A1; the proteins in Spots 3, 11–13 were assigned as hnRNP A3; and the proteins in Spot 7 and 10 were assigned as carcinoma associated antigen and SYNCRIP, respectively. The hnRNP A2/B1 and hnRNP A1 had been previously reported to bind G-rich telomeric DNA [20,21].

3.2. Confirmation of the identity for hnRNP A3

Analysis of the MALDI-TOF MS spectrum for Spot 11 in Fig. 1 revealed that the peptides could match 34% of human hnRNP A3 (Fig. 2A–C). To confirm the assigned identity of hnRNP A3, the peptide with *m/z* 1882.93 in Fig. 2A was further analyzed by TOF–TOF and the deduced peptide sequence (DTEEYNLR) that corresponds to hnRNP A3 was underlined in Fig. 2C. Based on these results, we confidently assigned the protein in Spot 11 as hnRNP A3. Finally, to confirm that hnRNP A3 is indeed specifically pulled down by the biotinylated G-3, biotinylated G-3, G-3S, C-3, NS, and ds G/C were employed as the affinity oligonucleotides in the pull-down assay, and the presence of hnRNP A3 and hnRNP A2/B1 in the supernatant (Sup) or pellet (Pellet) was analyzed by Western blot. As shown in Fig. 3, hnRNP A3 and hnRNP A2/B1 were selectively pulled down by the biotinylated G-3 but not by the G-3S, C-3, NS, or ds G/C.

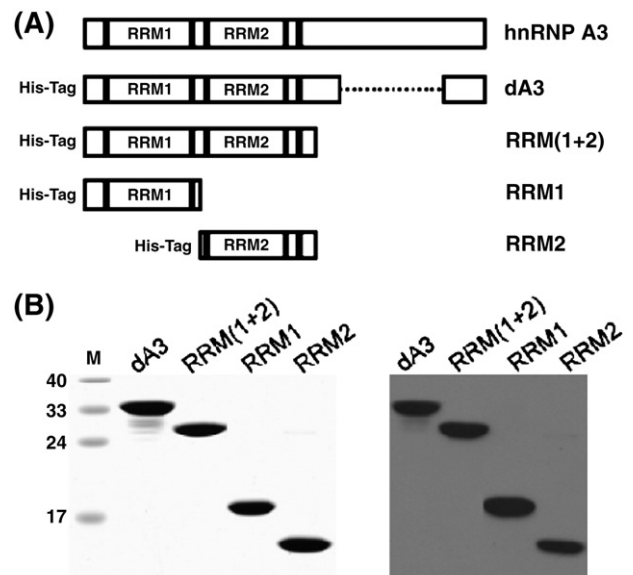


Fig. 4. Schematic presentation of the cloned derivatives of hnRNP A3 (panel A) and the purified proteins from these derivatives (panel B). In panel A, the various regions of hnRNP A3 that were cloned onto the expression plasmids were schematically depicted. The proteins expressed by these plasmids were purified by nickel column and separated in a 12% polyacrylamide gel containing SDS (SDS-PAGE). The proteins in the SDS-PAGE were identified by staining with Coomassie brilliant blue (left in panel B) or by Western blot with antibody against His-tag (right in panel B). The molecular mass markers (M) with the indicated mass in kilodaltons are shown in the left margin.

3.3. Cloning and biochemical characterization of hnRNP A3 and its derivatives

To investigate the DNA-binding properties of hnRNP A3, we have employed RT-PCR to clone the cDNA of hnRNP A3 into bacterial expression vector pET-15b (pET-15b/hnRNP A3). Despite that the initial pET-15b/hnRNP A3 contains the full-length cDNA of hnRNP A3, stable clones derived from the propagation of this plasmid in BL21(DE3)pLysS cells invariably were deleted in nucleotides 735–974. Therefore, the proteins isolated from the overexpression of this plasmid in BL21(DE3)pLysS cells were deleted for amino acids 245–335, which we named dA3 (Fig. 4A). The relative molecular mass (Mr) of purified dA3, RRM1, RRM2 and RRM(1+2), shown in Fig. 4B, was in agreement with the predicted values.

To address if dA3 possesses the telomere-specific DNA-binding activity, purified dA3 was first mixed with biotinylated G-3, and assayed by EMSA and pull-down method. As shown in Fig. 5A, dA3 could be pulled down by G-3, but not by G-3S. Incubation of biotinylated G-3 with increasing concentration of dA3 produced slowing moving complexes which could be competed by unlabeled G-3, but not by G-3S competitor (Fig. 5B). These results indicate that dA3 can bind directly to G-3 but not to G-3S. Next, the substrate specificity of dA3 was examined using EMSA. As shown in Fig. 5C, dA3 could only bind to G-rich single-strand DNA, but not C-rich strand, double-stranded telomeric DNA, or TS. Finally, we addressed what is the minimal telomeric repeat length that is required for the observed DNA binding by dA3. As shown in Fig. 5D, dA3 could bind to single-strand DNA containing two or more repeats of TTAGGG, but not to G-1 or NS.

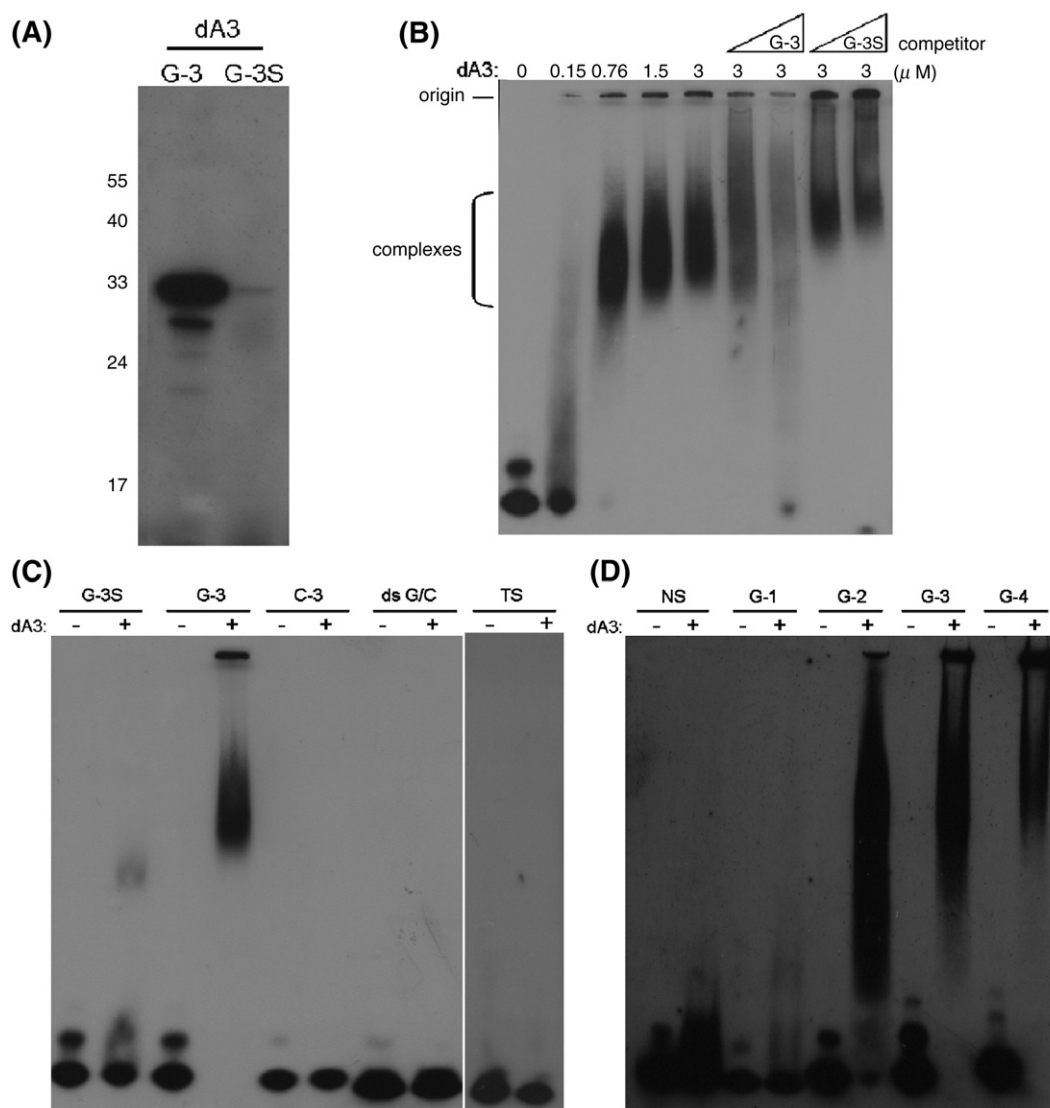


Fig. 5. DNA-binding activities of purified dA3. (A) Purified dA3 was mixed with biotinylated G-3 or biotinylated G-3S, and the pull-down proteins were analyzed by Western blot using antibody against His-tag. (B) Biotinylated G-3 was incubated with different concentrations of dA3 in the absence or presence of excessive non-labeled G-3 or G-3S. The DNA-binding activity of dA3 was assayed by EMSA. (C) Substrate specificity of dA3 was assayed by EMSA with the following DNA substrates: G-3, G-3S, C-3, TS and dsG/C. (D) The length of telomeric repeat required for the binding of dA3 was assayed by EMSA with non-binding sequence (NS) containing one to four TTAGGG repeats (G-1, G-2, G-3 or G-4).

3.4. RRM1 domain suffices for the DNA-binding activity of hnRNP A3

To address which functional domain is responsible for the observed binding to G-rich DNA by dA3, we analyzed the DNA-binding activity of RRM1, RRM2 and RRM(1+2) using both EMSA and pull-down method. As shown in Fig. 6A, RRM2 could not be pulled down by either G-3 or G-3S, while both RRM1 and RRM(1+2) could be pulled down by G-3. However, weak but detectable amount of RRM1 and RRM(1+2) was present in the pellets pulled down by G-3S. Incubation of biotinylated G-3 with increasing concentration of RRM1 or RRM(1+2), but not RRM2, produced slowing moving complexes (Fig. 6B and C). Although the mobility shift requires a higher concentrations of RRM1 as compared with RRM(1+2) or dA3 (Fig. 5B), these results, nonetheless, indicate that RRM1 suffices for the DNA-binding activity of hnRNP A3.

3.5. hnRNP A3 inhibits telomerase *in vitro*

The specific binding of hnRNP A3 to telomeric DNA raises the question that this protein may affect the synthesis of telomeric DNA by telomerase. To address this question, the effects of hnRNP A3 on the synthesis of telomeric DNA were examined *in vitro* by TRAP. When the dA3 was added to the reaction mixtures prior to the start of the assay by TRAP, the telomerase activity was greatly inhibited when the added dA3 was greater than 0.25–0.5 μ M (Fig. 7A). This inhibition of telomerase activity was also observed by the addition of RRM(1+2) or RRM1, but not RRM2 (Fig. 7A). The concentrations of RRM(1+2) and RRM1 required to inhibit telomerase were higher than that of dA3, which may reflect their relative activity to bind G-rich telomeric DNA as shown in Figs. 5 and 6.

To determine if the observed inhibition of telomerase activity may be at the step of PCR amplification, the telomerase in the nuclear extracts was allowed to synthesize telomeric repeats for

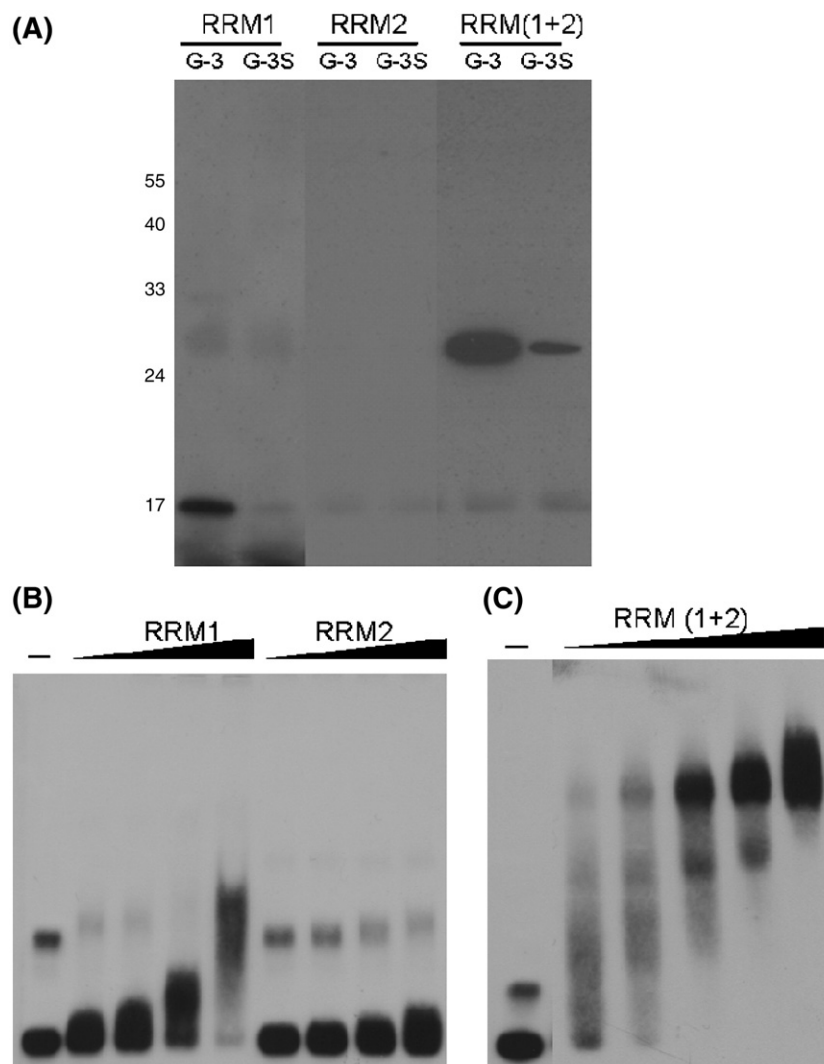


Fig. 6. DNA-binding activity of RRM1, RRM2 and RRM(1+2). The ability of RRM1, RRM2 and RRM(1+2) to bind telomeric DNA G-3 was assayed by pull-down method (A) and by EMSA (B and C). The conditions for pull-down assay and EMSA were as described in Materials and methods.

5, 10 or 15 min before the dA3 was added and amplified by PCR. As shown in Fig. 7B, delayed addition of dA3 also eliminated the detection of telomerase activity, indicating that dA3 interferes with the PCR amplification of the DNA products synthesized by telomerase. Finally, to determine if dA3 inhibits the step of extension by telomerase, dA3 was added to the extension mixtures at 0, 5, 10 or 15 min, and the DNA was extracted at the end of extension period before performing the PCR amplification. As shown in Fig. 7C, DNA products that contain extended telomeric repeats were not detected if dA3 was added at the start of the extension. However, DNA products

with extended telomeric repeats were readily detected if the addition of dA3 was delayed. Since dA3 did not bind to TS primer (Fig. 5C), and the lengths of telomeric repeats synthesized prior to the addition of dA3 appeared to correlate with the times of delayed addition (Fig. 7C), these results indicate that dA3 inhibits the extension by telomerase.

4. Discussion

In this work, we have employed affinity purification protocol combined with MALDI-TOF analyses to identify proteins that

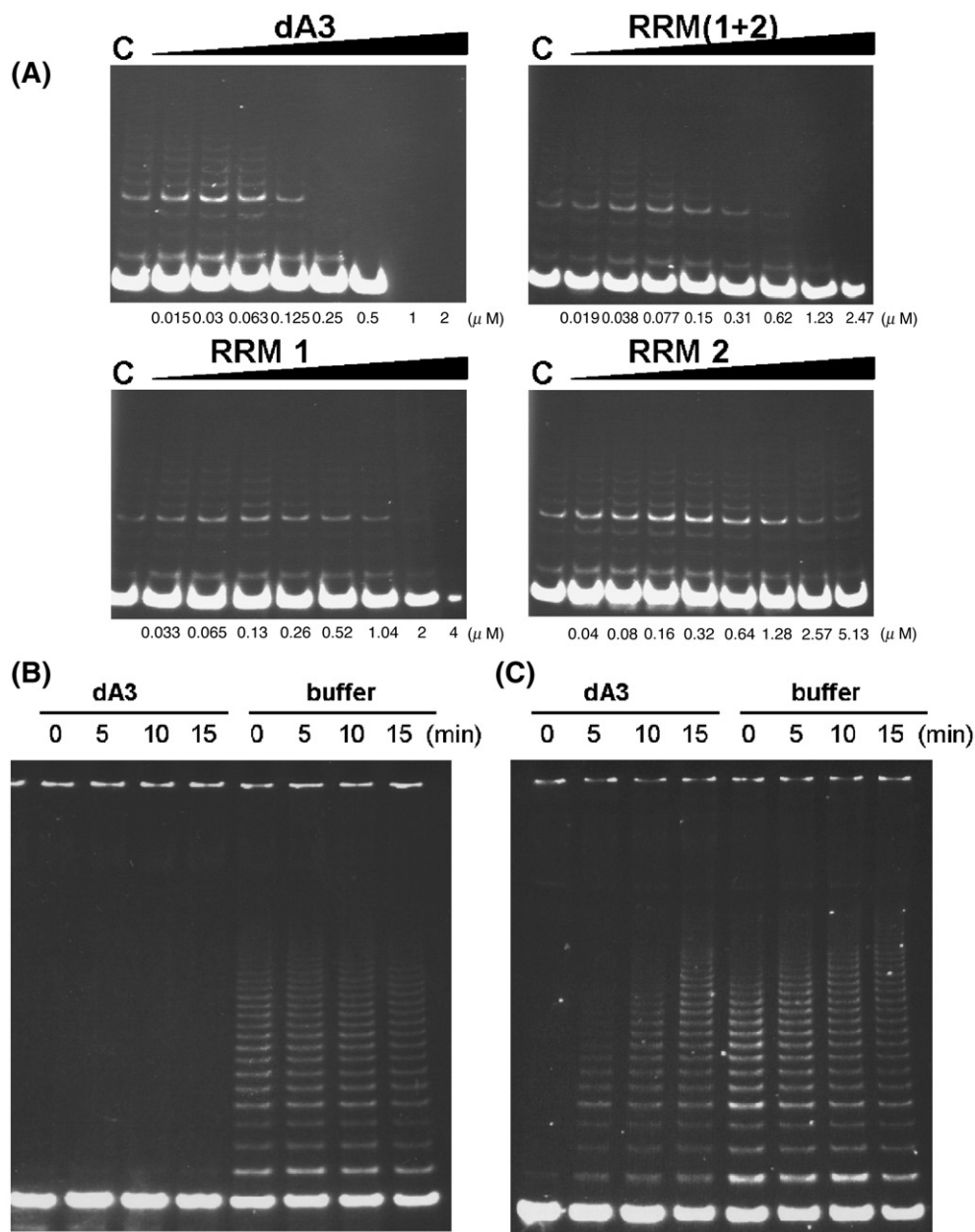


Fig. 7. Effects of hnRNP A3 derivatives on the telomerase activity *in vitro*. (A) Nuclear extracts were mixed with increasing concentrations of dA3, RRM1, RRM2 or RRM(1+2) and assayed for telomerase activity by TRAP. (B) Nuclear extracts were incubated with the primer TS for different lengths of time before adding dA3 to the reaction mixtures. The extended DNA products in the presence of added dA3 were subsequently amplified by PCR and resolved in the neutral polyacrylamide gel. (C) Nuclear extracts were incubated with the primer TS for different lengths of time before adding dA3 to the reaction mixtures. The extended DNA products in the reaction mixtures were extracted with phenol before subsequent amplification by PCR. The PCR products were then resolved in the neutral polyacrylamide gel.

bind to G-rich strand of telomeric DNA. Similar to the findings by Ishikawa et al. [22], the majority of the identified proteins are hnRNPs. Among the five putative proteins identified in this study, hnRNP A2/B1 and hnRNP A1 had been shown to bind G-rich telomeric DNA with high specificity [20,21]. hnRNP A3 was recently identified in the pull-down with a telomeric oligonucleotide [23], although its specificity to bind telomeric DNA had not been demonstrated. The other two putative proteins, carcinoma-associated antigen and SYNCRIP, were not described previously. Surprisingly, other known G-rich strand telomere-binding proteins, such as Pot1 and hTERT, could not be identified in the present approach. Presumably, the abundance of hnRNP A1, hnRNP A2/B1, and hnRNP A3 in the nuclear extracts are far greater than that of Pot1 and hTERT, thus overshadowing the ability to detect scarce telomere-binding proteins.

Here we have cloned the cDNA of hnRNP A3 onto bacterial expression vector to study the biochemical properties of purified hnRNP A3. Much to our surprise, however, the purified hnRNP A3 had an apparent molecular mass that was smaller than the predicted value. Analysis of the DNA sequence in the stable clones derived from the propagation of pET-15b/hnRNP A3 in BL21(DE3)pLysS cells revealed that the *hnRNP A3* gene was deleted at nucleotides 735–974. Despite many attempts to modify the conditions of propagating this plasmid in BL21 (DE3)pLysS cells, we had been unable to obtain intact hnRNP A3 protein so far. Examination of the region that is deleted in the stable clones reveals that there are two direct repeats with high homology at positions 718–734 and 958–974 of the *hnRNP A3* gene. An active recombination between these sites is likely to account for the observed deletion in this region.

Despite our inability to obtain intact hnRNP A3 for DNA-binding studies, we have shown that the hnRNP dA3 possesses the DNA-binding activity which is specific to the G-rich strand,

but not to the C-rich strand or double-strand of telomeric DNA (Fig. 5A–C). The amino acids deleted in the hnRNP dA3 were in the C-terminal Gly-rich region, which have been reported to influence the binding affinity of the A/B group of hnRNP proteins to RNA [24] but not to telomeric DNA [23,25]. Here, we presented evidence that the RRM1 domain but not the RRM2 domain of hnRNP A3 is essential for the telomeric DNA-binding activity. Nonetheless, the RRM2 appears to play a role in facilitating the DNA binding, since RRM(1+2) had a greater DNA-binding activity than RRM1 (Fig. 6). The findings that the RRM1 of hnRNP A1 (20), A2/B1 (23) and A3 (Fig. 6) can bind specifically to telomeric DNA suggest that such a DNA-binding activity may be common characteristics of RRM1+RRM2 of A/B groups of hnRNP proteins. However, our results for the binding of G3 telomeric DNA to individual RRM1s differ from the previous observations with hnRNP A2, which showed some binding to RRM2 but no binding to RRM1 [23]. In a similar study for the binding of a decameric telomeric repeat, the RRM1 of hnRNP A1 was shown to display strong binding, the RRM2 displayed a weaker binding and the RRM concatamer (UP1) had the strongest association [20]. Therefore, hnRNP A3 appears to bear more resemblance to hnRNP A1 in the telomeric DNA binding. A comparison of RRM1 from hnRNP A1, A2 and A3 have indicated that an identity of 90.3% between A1 and A3, whereas the identity between A2 and A3 was 77.8% (Fig. 8). There are a total of 9 amino acids that are conserved in A1 and A3, but not in A2 (underlined in Fig. 8). One or more of these sites may be potentially critical for the telomeric DNA-binding activity of RRM1.

It is interesting to note that the major G-rich strand telomere-binding proteins identified in the nuclear extracts are hnRNPs ([22] and this study). These hnRNPs were originally found to associate with pre-mRNA and thought to participate in pre-mRNA processing, alternative splicing, transcriptional regula-

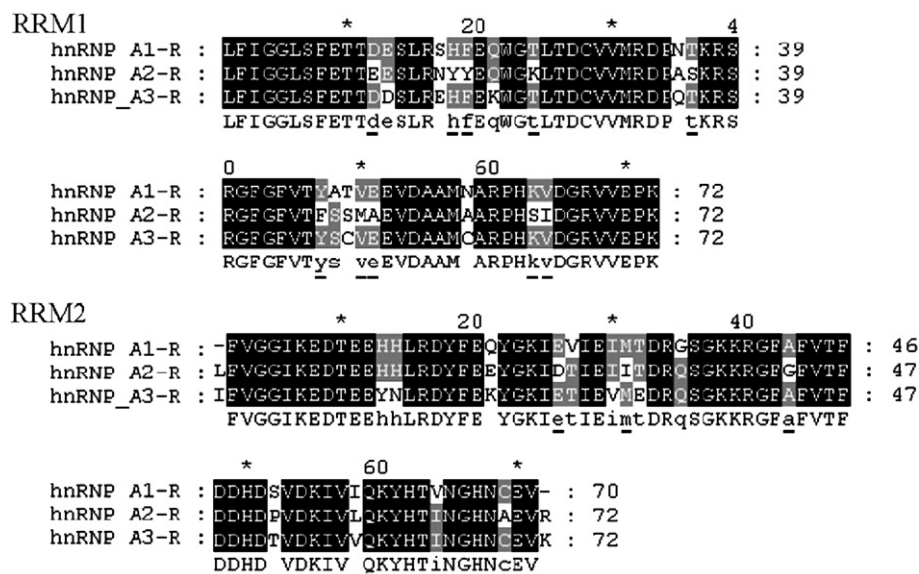


Fig. 8. Amino acid sequence comparison for the RRM1 and RRM2 of hnRNP A1, A2, and A3. The amino acids that are conserved in all hnRNPs are marked with capital letters in the bottom. The amino acids that are conserved in A1 and A3, but not in A2, are underlined.

tion, or mRNA transport [26]. The findings that many of these hnRNPs bind to G-rich strand telomeric DNA suggest that these proteins can also participate in telomere function. Although there is no report that hnRNPs may be a structural component of telomere, several hnRNPs have been shown to interact with telomerase, to protect telomeric DNA from nuclease attack and to affect the synthesis of telomeric repeats *in vitro* [14,20,25,27,28]. In fact, a role of hnRNP A1 in telomere maintenance *in vivo* has been convincingly documented [29] and a stimulatory effect of telomerase activity by hnRNP A1 has been reported [28]. Although the DNA-binding property of hnRNP A3 is similar to hnRNP A1, our finding for an inhibition of the telomerase extension by hnRNP dA3 *in vitro* (Fig. 7) suggests that hnRNP A3 may function negatively in telomere maintenance *in vivo*.

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